



## REMARKS

The examiner has objected to claim 1 because the named bacteria is not italicized. Accordingly, the named bacteria has now been italicized as required by the examiner.

The examiner has also objected to claims 1 and 25 because these claims lack the article "A" as the first word in the claim. Accordingly, claims 1 and 25 have been amended so that these claims now begin with the phrases "A purified" and "A vaccine" respectively as requested by the examiner.

The examiner notes in item 9 of the office action that the trademarked product "Tween 20" on page 7, line 12 and page 13, line 25 should be properly respected. Accordingly, applicant has made an appropriate amendment to this term on pages 7 and 13 of the specification. In this regard it is to be noted that the trademarked material is now identified with capital letters as suggested by MPEP § 608.01V.

The examiner notes that figure 6 is improperly labeled and suggests that the first page of the drawings should be labeled as figure 6A and the second, third, fourth and fifth continuing pages should be labeled as figure 6B, 6C, 6D and 6E respectively. The examiner further notes that the figure descriptions in the specification should correspond to the re-labeled drawings and references to these figures throughout the specification should be amended. Accordingly, applicant submits herewith a proposal for amending the figures in accordance with the examiner's suggestion. In addition, an appropriate amendment to the specification has been made so that the term "figure 6" now reads as: "Figures 6A-6E".

The examiner has objected to the abstract because the abstract contains the word "said" which should be avoided. Accordingly, applicant has made the appropriate correction to the abstract.

The examiner has rejected claims 1-25 under 35 U.S.C. § 102(b) as being anticipated by Timmerman. In rejecting the claims the examiner urges that the protein disclosed by Timmerman is necessarily the same as the claimed purified protein or polypeptide because of a similarity in the molecular weight between Timmerman's protein and the claimed purified protein. Applicant has carefully considered this rejection but it is most respectfully traversed for the reasons discussed below.

The examiner notes on page 3 of the office action that claim 1 is not limited to a protein having "insignificant fibrinogen binding activity". Thus it appears that the examiner may have misunderstood the invention since the purified protein or polypeptide of the present invention has significant fibrinogen binding activity. Accordingly, claim 1 has been amended to recite that the protein or polypeptide has substantial fibrinogen binding activity to clarify this aspect of the invention.

Furthermore, and more importantly, claim 1 is directed to a purified protein or polypeptide. Timmerman does not disclose or suggest any purified protein or polypeptide.

Furthermore, the examiner's rejection is based upon his conclusion that the protein disclosed by Timmerman et al. is inherently the same protein of claim 1 which has fibrinogen binding activity. As noted in applicant's last response, the two proteins cannot be the same because Timmerman's protein adheres to biomaterial and mediates attachment to polystyrene (see the article, p. 4187, left column, just before "Materials and Methods"). In other words the protein disclosed by Timmerman binds to plastic.

In contrast, it is noted in applicant's specification that the protein of the invention has no significant plastic binding activity (see table 2 in the specification). Thus, it is self evident from the above that contrary to the examiner's observation, the protein disclosed by Timmerman et al. is not the same as the claimed protein and therefore, there is absolutely no basis for the examiner to conclude that Timmerman's protein would inherently have fibrinogen binding activity.

Applicant submits that Timmerman does not disclose any purified protein. In this regard it is to be noted that in Timmerman's abstract is disclosed "immunoblotting of cell wall polypeptides of strain 354 revealed strong reactivity with a 200- to 220-kDa **band** and a weaker reaction in the 100- to 110-kDa **range**" (emphasis added). Clearly the bandwidth and range indicate that a single protein has not been purified. Furthermore, it is noted on p. 4189, left column, lines 9-12 that "both MAbs reacted strongly with two sharply demarcated protein bands of approximately 220 kDa, while a considerably weaker reactivity was noted with protein bands in the 110-kDa range". In addition, it is stated on p. 4191, left column, second paragraph, that "in the present study we confirmed and expanded upon these studies by demonstrating that a **protein complex** of approximately 220 kDa contains an antigen apparently involved in the adherence of *S. epidermidis* 354 to a polystyrene substratum *in vitro*."

It is therefore clear that contrary to the examiner's observation, Timmerman does not disclose any purified protein.

Applicant further submits that the work by Timmerman has absolutely no relevance to applicant's invention for the following reasons. Firstly, the ability of Timmerman's proteinaceous preparation to bind fibrinogen has never been tested experimentally nor do the authors even speculate that the material should bind fibrinogen.

Concerning the molecular weight, the materials described by Timmerman are claimed to be composed proteins in the 200-220 kDa range and in the range of 100-110 kDa. The examiner seems to be of the opinion that if a protein obtained from a specific organism has a determined molecular weight and a specified function, then all other proteins from the same organism having approximately the same molecular weight would inherently have the same function. Applicant submits that one skilled in the art would find no basis for making this conclusion. Clearly, one skilled in the art would have no basis to believe that the protein disclosed by Timmerman would inherently have the same type of fibrinogen binding activity as found in applicant's claimed protein.

A comparison of molecular weights made on SDS polyacrylamide gels is not at all a proof that, for example, two unknown proteins should be identical or functionally related only on the basis of their apparent molecular weights. In this case it is also most likely that the 100-110 kDa fraction in the preparation by Timmerman is actually a degradation product of the 200-220 kDa fraction. This is also strengthened by the fact that the monoclonal antibodies used (see figure 1 of Timmerman) binds both to the 200-220 kDa and 100-110 kDa fractions. Since a monoclonal antibody by definition reacts with one epitope, the binding to both fractions would mean that both fractions share epitopes and thus the 100-110 kDa fraction is likely to be a truncated product of the 200-220 kDa fraction.

In view of the above, it is clear that the examiner has no factual basis for concluding that the protein disclosed by Timmerman is the same as the claimed protein and therefore, it follows that there is no basis to conclude that Timmerman's protein

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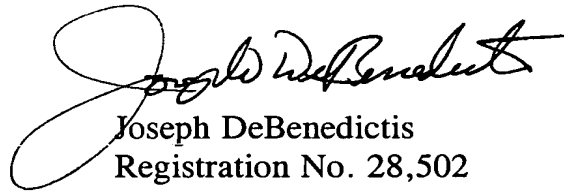
would likewise have fibrinogen binding activity. Accordingly, the rejection is unfounded and must be withdrawn.

Respectfully submitted,

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**VERSION WITH MARKINGS TO SHOW CHANGES MADE**

MARKED UP VERSION OF SPECIFICATION:

Section labeled "Short description of the figures" beginning on page 2, line 1 and ending on page 3, line 1:

Short description of the figures

The invention will be described in closer detail in the following, with support of the enclosed examples and figures, in which

Fig. 1 shows the adherence values as a function of fibrinogen coating concentration for the *S. epidermidis* strains 2, 19 and JW27 (Example 1A),

Fig. 2 shows percent inhibition for antibodies against fibrinogen, compared to antibodies against fibronectin (Example 1B),

Fig. 3 shows percent inhibition as a function of competing fibrinogen concentration (Example 1C),

Fig. 4 shows the protease sensitivity of adherence to fibrinogen (Example 1D),

Fig. 5 shows the inhibition of adherence by LiCl extract (Example 1E),

Figures 6A-6E show [Fig. 6 shows] the complete nucleotide sequence of the *fig* gene from *S. epidermidis* strain HB and the deduced amino acid sequence of the encoded protein (SEQ ID NO:14). A putative ribosomal binding site (RBS) is underlined and a possible transcription termination hairpin loop is double underlined. A putative signal sequence (S) is indicated with an arrow and the translational stop codon with an asterix. The start of the non-repetitive N-terminal region called A, harbouring the fibrinogen binding activity is indicated by an arrow. R indicates the highly repetitive region. The motif LPXTG involved in cell wall anchoring is indicated in bold characters and the membrane-spanning region is marked M (Example 3 ),

Fig. 7 shows a schematic drawing comparing the fibrinogen binding protein FIG

of *S. epidermis* and the clumping factor (ClfA) of *S. aureus*. The similarity, (%), of corresponding regions in the proteins is indicated in the figure between the two protein bars. S is the signal sequence; A, the non-repetitive region harbouring the fibrinogen binding activity, R, the diamino acid residue repeat region; W the region proposed to be involved in cell wall anchoring and M, the transmembrane domain. The numbers indicated refer to the amino acid positions in respective proteins as shown in [Figure 6] figures 6A-6E and 7 and in reference (McDevitt et al., 1994) (Example 3),

Fig. 8 shows how GST-FIG fusion protein is captured to fibrinogen in a dose dependent way (Example 10),

Fig. 9 shows the decrease of bacterial binding as a function of GST-FIG fusion protein, GST or FIG (Example 11),

Fig. 10 shows the relative adherence as function of serum dilution for two pre immune sera and a serum against GST-FIG and FIG, respectively (Example 12), and

Fig. 11 shows the relative bacterial adherence as a function of serum dilution for, on one hand, pre immune serum and, on the other hand, serum against GST-FIG (Example 12).

Replacement paragraph for page 7, beginning at line 8 and ending at line 21:

Fibrinogen was dissolved in PBS at 10 mg/ml and added in serial 3-fold dilution to microtiter wells (Nunc), from top to bottom. The plates were incubated overnight at room temperature (RT). To cover uncoated plastic sites the plates were coated with 2% bovine serum albumin for 1 hour at 37°C. The plates were washed with PBS with 0.05% [Tween 20] TWEEN 20 (PBST). Next, bacteria were added in serial 2-fold dilution in PBST, from left to right, to the fibrinogen coated microtiter plates. Bacterial adherence was allowed for 2 hours at 37°C or at 4°C overnight. Non-adherent bacteria were washed off and the bound bacteria were air-dried. The

crosswise dilution of both fibrinogen and bacteria allows estimation of bacterial binding both as a function of fibrinogen concentration and of amount of bacteria. Determination of bacterial adherence was done by optical reading using a microtiter plate reader at A 405. The turbidity and light scatter caused by bound bacteria results in a reading ranging from 0.00 to 0.20. An example of adherence values as a function of fibrinogen coating concentration is shown in Figure 1 for three different strains (2, 19 and JW27). These conditions for adherence determination were used in the following experiments.

Replacement paragraph for page 10, beginning at line 6 and ending at line 29.

To obtain the missing 5' and 3' end of the *fig* gene a Southern blot analysis was performed using chromosomal DNA from strain HB digested with various restriction enzymes. The probe was prepared as follows; two oligonucleotides (5'CAACAACCATCTCACACAAC3' which is SEQ ID NO:1 and 5'CATCAAATTGATATTTCCCATC3' which is SEQ ID NO:2) were used to PCR amplify a ~1.3kb fragment from the insert of pSE100. The PCR generated fragments were 32P-labeled using random priming. After hybridisation using stringent conditions the NC-filter was washed and subjected to autoradiography. The result showed that the XbaI cleavage gave a single band in size of ~6 kb. The corresponding fragment was subsequently ligated into XbaI digested pUC18 vector. After transformation clones harbouring the ~6kb XbaI-fragment were identified by colony hybridisation using the same probe as in the Southern blot experiment. One such clone, called pSE101 was chosen for further studies. DNA sequence analysis showed that the *fig* gene consist of an open reading frame of a 3291 nt, encoding a protein, called FIG of 1097 aa with a calculated molecular mass of ~119 kDa (Figures 6A-6E) [(Figure 6)]. The FIG protein consist of several typical features found among Gram-positive cell surface bound proteins, like a N-terminal signal sequence and a C-terminal aa motif LPDTG,



followed by a stretch of 17 hydrophobic aa ending in a stretch of charged aa (Figure 6). Following the signal sequence, there is a region, called A of 773 aa. The insert of pSE100 contains the sequence corresponding to residue 75 to 656 of the A region (Figure. 7). The A region is followed by a highly repetitive region of 216 aa composed of tandemly repeated aspartic acid and serine residues, called R (figures 6A-6E and 7) [(Figure 6 and 7)]. The dipeptid region consist of an 18 bp sequence unit (consensus of GAX TCX GAX TCX GAX AGX which is SEQ ID NO:3) repeated 36 times. The 18 bp sequence is almost maintained perfect throughout the whole R region except for the second unit which is truncated, consisting of only 12 of the 18 bp and the 3' end of the region where the consensus sequence is slightly disrupted (units 32, 34 and 36). The changes in the later units also result in an amino acid exchange which disrupt the DS repeat.

Replacement paragraph for page 13, beginning at line 12 and ending at page 14, line 5.

#### Example 5: Western blot experiment

*E. coli* cells of strain TG1 and MC1061 containing pSE100 were grown in LB (containing ampicillin and glucose) over night at 37°C. The next morning the cells were harvested by centrifugation, resuspended in LB (containing ampicillin, glucose and 0.1 M IPTG and further incubated at 37°C. Twelve hours later the cells were harvested by centrifugation and both the cells and the supernatant were taken care of. Four volumes of acetone were added to the supernatant and the resulting precipitate was collected by centrifugation, air-dried and resuspended in ice-cold PBS. Prior to electrophoresis the cells and the precipitate from the supernatant were resuspended separately in a sample buffer containing 2.5 % SDS and 5 % beta-mercaptoethanol and boiled for two minutes. After denaturation the samples were analysed run under reducing conditions using the PHAST-system (Pharmacia) on a 8-25 % gradient gel

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using SDS-buffer strips. After the electrophoresis was completed a NC-filter previously soaked in PBS was put on the gel and the temperature raised to 45°C. After ~45 minutes the NC-filter was wetted with 1 ml PBS, gently removed and placed in 15 ml PBS containing 0.1 % [Tween 20] TWEEN 20 solution (PBST 0.1 %) for 30 minutes in RT (under gentle agitation and with two changes of PBST 0.1 % solution). After the last change of PBST 0.1 % fibrinogen was added to a final conc. of 20ng/ml and the filter was incubated for four hours at RT under gentle agitation. The filter was subsequently washed for 3x10 minutes using PBST0.1 % and HRP-conjugated rabbit anti-human fibrinogen antibodies (DAKO code A 080, diluted 1:500 in PBST 0.1 %) were added and the filter was incubated for 1 hour at RT under gentle agitation. After washing the filter 3x10 minutes using PBST 0.1 % the bound fibrinogen was visualised by transferring the filter to a solution containing a substrate for the horse radish peroxidase (6 ml 4-chloro-1-naphtol (3 mg/ml in methanol) + 25 ml PBS + 20  $\mu$ l H<sub>2</sub>O<sub>2</sub>). The result showed that a fibrinogen binding protein was found in both types of samples (cells and growth media) in both *E. coli* cells harbouring pSE100, while no such protein was found in the control cultures of *E. coli* TG 1 and MC1061. The fibrinogen binding protein expressed from the pSE100 was in the approximate size as expected from the deduced amino acid.

Replacement paragraph for page 16, beginning at line 1 and ending at line 17.

#### Example 9: Production of GST-FIG

By polymerase chain reaction, a DNA fragment was amplified encoding a portion of the fibrinogen binding protein. Upper primer was GCGGATCCAATCAGTCAATAAACACCGACGAT (SEQ ID NO:8) and lower primer was CGGAATTCTGTTCGGACTGATTTGGAAGTTCC (SEQ ID NO:9). Amplification was done for 30 cycles at 94°C 30 seconds, 60°C 30 seconds, 72°C 2 minutes beginning with 94°C for 4 minutes and ending with 72°C for 4 minutes. The

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amplified fragment was digested with EcoRI and Bam HI. Plasmid pGXT-4T (Pharmacia, Uppsala, Sweden) was digested with EcoRI and Barn HI, mixed with the digested fragment and the mixture ligated using T4 DNA ligase according to standard procedures. The ligated DNA was transformed into *E. coli* strain TGI. A transformant was isolated with a plasmid encoding a fusion protein composed of glutathione thio transferase and fibrinogen binding protein. The protein was purified with the vector plesmid according to Pharmacia's instructions. The purified GST-FIG protein was subjected to Western affinity blot. It was run on polyacrylamide gel electrophoresis, transferred to nitrocellulose paper by passive diffusion, the paper treated with fibrinogen (5  $\mu$ g/ml) for 2 hours at room temperature, followed by rabbit anti fibrinogen antibodies conjugated to HRP. A band corresponding to a molecular weight of approx. 100 kDa was seen. Omitting fibrinogen in a control experiment displayed no band.

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MARKED UP VERSION OF ABSTRACT:

ABSTRACT OF THE DISCLOSURE

A new fibrinogen binding protein or polypeptide originating from coagulase negative staphylococci, biotechnological methods for producing [said] the protein or polypeptide having fibrinogen binding activity and a recombinant DNA molecule coding for [said] the protein (or fragments thereof), and micro-organisms (including viruses) containing this recombinant DNA molecule. The present invention further comprises the therapeutic and diagnostic use of [said] the protein and/or DNA, e.g., a diagnostic kit for determining the presence and/or type of coagulase negative staphylococci and a vaccine composition, comprising [said] the protein or DNA.

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MARKED UP VERSION OF CLAIMS:

1. (Thrice amended) A purified *Staphylococcus epidermidis* [Purified staphylococcus epidermidis] protein or polypeptide having substantial fibrinogen binding activity[,].

25. (Amended) A vaccine [Vaccine] composition including a protein according to claim 1.